FBS12- PCR Amplification Using the Identifiler Plus Kit

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1. Scope

1.1. This procedure is used to amplify DNA using the AmpFlSTR® Identifiler® Plus Kit.

2. Background

2.1. To establish the practices for documenting the examination of evidence to conform to the requirements of the Department of Forensic Sciences (DFS) Forensic Science Laboratory (FSL) *Quality Assurance Manual*, the accreditation standards under ISO/IEC 17025:2005, and any supplemental standards.

2.2. In order to ascertain if a profile(s) is present in a sample's extract, the purified DNA must be replicated and labeled for detection. The AmpFlSTR® Identifiler® Plus Kit is a short tandem repeat (STR) multiplex kit which utilizes the enzymatic process of Polymerase Chain Reaction (PCR) to amplify 15 specific DNA locations and one gender marking position. These 16 loci include the 13 core CODIS loci. Each kit is comprised of a fluorescent dye-labeled locus-specific primer set, PCR master mix (which includes the enzyme DNA polymerase), 9947A Positive Control DNA and allelic ladder.

3. Safety

3.1. Wear personal protective equipment (e.g., lab coat, gloves, mask, eye protection), when carrying out standard operating procedures.

3.2. Read Material Safety Data Sheets to determine the safety hazards for chemicals and reagents used in the standard operating procedures.

4. Materials Required

4.1. AmpFlSTR® Identifiler® Plus Kit – Primers, Reaction Mix with DNA polymerase enzyme

4.2. 9947A Positive Control DNA

4.2.1. Note: It is important to minimize the number of freeze-thaw cycles for the kit reagents. Keep the kits protected from direct exposure to light. Excessive exposure can affect fluorescent probes. Each lot of kits must be evaluated prior to use. See QSOP 33 for information regarding the procedure for evaluation. Amplification reagents must be stored separately from the evidentiary samples.

4.3. 0.5ml thin-walled reaction tubes (individual or strips)/strip caps or 96-well plate/strip caps/adhesive seal

4.4. 32mg/ml BSA

4.5. TE Buffer

4.5.1. NOTE: Never use solutions directly from the stock bottles. Use Reagent SOPs for preparation and labeling instructions.

5. Standards and Controls

5.1. The amplification Positive (9947A) and Negative Controls are incorporated into the sample set following all other samples. One set of controls may be tested with each sample set. Results are printed after analyzed.

5.1.1. 9947A is amplified as a Positive Control. This control is used to evaluate the performance of the amplification and subsequent typing procedures. See the results interpretation guidelines for the known profile that is generated from 9947A.

5.1.2. TE Buffer is amplified as the Negative Control. This control contains all of the chemical components of the amplification reaction in addition to TE Buffer and should exhibit no profile.

5.1.3. Extraction reagent blanks must be amplified at a volume equal to the highest preparation volume of any sample in its associated batch. In other words, the same concentration conditions as the forensic samples containing the least amount of DNA. This control contains all of the chemical components of both the extraction and amplification reactions and should exhibit no profile. Furthermore, the reagent blank must be amplified using the same primers and instrument model as the forensic sample(s) it is associated with.

6. Calibration

6.1. Not applicable

7. Procedures

7.1. Sample Set-up Calculations:

7.1.1. Using the estimated quantities of DNA obtained from the Quantifiler® Duo DNA Quantification Kit, calculate the number of microliters (and/or the necessary dilution) to be added to the amplification reaction in order to obtain a concentration of 0.05-0.10 ng/µl. The combined volume of TE buffer and sample DNA should equal 10µl. A typical target amount of DNA comprised within the 10 µl per validation procedures is 0.5 – 1.0ng.

7.1.2. The following chart is an example which can be used to calculate a sample to a 0.5ng target:

If you are preparing the…	*Then…*
DNA sample and the concentration is ≤0.05ng/µl	*Add 10µl of sample to the PCR tube/well*
DNA sample and the concentration is > 0.05/µl	*Dilute a portion of the sample with TE buffer so that only 0.5 ng of total DNA is in a volume of 10µl.*
Positive Control	*Add 5 µl 9947A to the PCR tube/well*
Negative Control	*Add 10 µl TE Buffer to the PCR tube/well*

7.1.3. Record this information on the Identifiler Plus Amplification Setup Worksheet. This worksheet will document how a sample was amplified and direct the set up process.

7.1.3.1. Note: Alternatively the Dilution Calculation Worksheet may be utilized to aid in the calculations.

7.2. Master Mix Preparation:

7.2.1. Calculate the amount of each component necessary to prepare the Master Mix.

# of Samples x 10 µl Master Mix

# of Samples x 5 µl Primer Set

7.2.2. Record the values on the Identifiler Plus Amplification Setup Worksheet.

7.2.2.1. Note: An additional two samples may be added to each calculation to account for volume lost during pipetting.

7.2.3. Obtain the following components from refrigerated storage: Reaction Mix, Primers, and 9947A.

7.2.4. Vortex and pulse spin all reagents. Record the appropriate lot numbers and expiration dates on the Identifiler Plus Amplification Setup Worksheet.

7.2.5. Obtain a 1.5 ml or 2.0 ml tube and label as Master Mix. (If amplifying a large quantity of tubes, the master mix may be prepared in a V-bottom basin.)

7.2.6. Add the pre-determined amount of Master Mix and Primer Mix to a tube (or V-bottom basin).

7.2.6.1. Note: Additional BSA may be added at the discretion of the analyst. For those samples requiring BSA, add 0.125µl of 32mg/ml BSA per sample to the mix.

7.2.7. Vortex and pulse spin. If prepared in a V-bottom basin, tip the basin from side to side to thoroughly mix. Store at 4°C until ready to aliquot. It is highly recommended to aliquot **IMMEDIATELY** after preparation.

7.3. Sample Distribution:

7.3.1. Allow the sample extracts to equilibrate to room temperature. Vortex and spin all tubes.

7.3.2. Obtain and label an appropriate quantity of 0.5ml thin-walled amplification tubes or a 96-well plate.

7.3.3. Place the tubes/plate in an appropriate retainer for stability.

7.3.3.1. Note: The retainers should be used for sample transport from the pre-amplification laboratory to the post-amplification laboratory **ONLY**. Prior to re-entry to the pre-amplification laboratory, all retainers must be soaked in 10% bleach, rinsed with diH20 and thoroughly dried. Alternatively, the retainers can be irradiated with UV light in a hood for 2 hours.

7.3.4. Aliquot 15 µl mix into each sample's amplification tube/well.

7.3.5. Following the Identifiler Plus Amplification Setup Worksheet, aliquot the calculated volume of neat extract/diluted extract and/or TE Buffer to each sample's associated tube or well. The 9947A Positive Control and the TE Buffer Negative Control, in that order, will be the last two samples to be added to the batch.

7.3.5.1. All tubes/wells should now contain a total volume of 25 µl.

7.3.6. Cap the tubes/seal the tray. Make sure that the caps/seal are secure.

7.3.7. Vortex and centrifuge.

7.4. Thermal Cycler:

7.4.1. Transport the amplification tray containing the tubes or the 96-well plate to the thermal cycler.

7.4.2. Load the samples onto the thermal cycler. Gently push the tubes/plate completely down into the heat block. Pull the lid closed over the samples until it clamps.

7.4.3. Turn on the thermal cycler. Select and start the appropriate thermal cycling program. The method on the screen should correspond to the following:

HOLD	*95°C*	*11 minutes*
CYCLE	*94°C*	*20 seconds*
	59°C	*3 minutes*
Repeat for 28 or 29 total cycles		
HOLD	*60°C*	*10 minutes*
HOLD	*4°C*	*forever*

7.4.3.1. Note: If the method on the screen does not correlate to the method outlined, refer to the "Creating and Editing Methods" section of the User's Manual for additional information on programming the instrument.

7.4.3.2. OPTIONAL: Thermal cycler may be preheated.

7.4.4. Press Start.

7.4.5. When amplification is complete, the samples can sit at 4°C (in the thermal cycler) for up to 24 hours. Pulse spin tubes/plate after removal and freeze at -20°C or proceed to preparation for the analysis (DSOP 10).

8. Sampling

8.1. Not applicable

9. Calculations

9.1. The following chart is an example which can be used to calculate a sample to a 0.5ng target:

If you are preparing the…	*Then…*
DNA sample and the concentration is ≤0.05ng/µl	*Add 10µl of sample to the PCR tube/well*
DNA sample and the concentration is > 0.05/µl	*Dilute a portion of the sample with TE buffer so that only 0.5 ng of total DNA is in a volume of 10µl.*
Positive Control	*Add 5 µl 9947A to the PCR tube/well*
Negative Control	*Add 10 µl TE Buffer to the PCR tube/well*

10. Uncertainty of Measurement

10.1. When quantitative results are obtained, and the significance of the value may impact the report, the uncertainty of measurement must be determined. The method used to determine the estimation of uncertainty can be found in the *FSL Quality Assurance Manual – Estimation of Uncertainty of Measurement (Section 5.4.6).*

11. Limitations

11.1. Given that the quantity reported by Real-Time PCR is an estimation, the amount of DNA added to an amplification reaction may be adjusted after detection to improve the quality of the profile.

11.2. All questioned and reference specimens must be processed separately. If questioned and reference specimens are on the same tray, the samples must be separated by the positive and negative controls associated with them.

11.3. For amplification troubleshooting procedures, refer to AB User's Manual. The following strategies can also be used:

11.3.1. Re-amplify with BSA.

11.3.2. Dilute DNA extract.

11.3.3. Concentrate DNA extract

11.4. If -A peaks are observed, the samples can be re-amplified using less DNA or the samples may be placed back into the thermal cycler for an additional extension step.

11.5. Once a case is complete, preserve the amplified DNA product for those samples in which the DNA extract and/or stain material was consumed during analysis. If sufficient stain material and/or extract remains for additional testing, the amplified product can be discarded. If the extract was consumed, the amplification product and its corresponding controls shall be maintained frozen. Any amplification product that is maintained by the laboratory shall be documented in the examination documentation.

11.6. The fluorescent dyes attached to the primers are light-sensitive. Store all the samples away from light and minimize the time in which these samples are exposed during analysis.

12. Documentation

12.1. Identifiler® Plus Amplification Setup Worksheet

13. References

13.1. Applied Biosystems. AmpFlSTR® Identifiler® Plus PCR Amplification Kit User's Manual.

13.2. Applied Biosystems. GeneAmp® PCR System 9700 User's Manual Set.

13.3. Budowle, B. STR allele concordance between different primer sets: a brief summary. Profiles in DNA (2000) 3: 10-11.

13.4. Budowle, B., Defenbaugh, D.A., and Keys, K.M. Genetic variation at nine short tandem repeat loci in Chamorros and Filipinos from Guam, Legal Medicine (2000) 2: 26-30.

13.5. Budowle, B., Masibay, A., Anderson, S.A., Barna, C., Beiga, L., Brenneke, S., Brown, B.L., Cramer, J., DeGroot, G.A., Douglas, D., Duceman, B., Eastman, A., Giles, R., Hamill, J., Haase, D.J., Janssen, D.W., Kupferschmid, T.D., Lawton, T., Lemire, C., Llewellyn, B., Moretti, T., Neves, J., Palaski, C., Schueler, S., Sgueglia, J., Sprecher, C., Tomsey, C., and Yet, D. STR primer concordance study, Forensic Science International (2001) 124: 47-54.

13.6. Moretti, T.R., Baumstark, A.M., Defenbaugh, D.A., Keys, K.M., Smerick, J.B., and Budowle, B. Validation of short tandem repeat (STRs) for forensic usage: Performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples, Journal of Forensic Sciences (2001) 46: 647-660.

13.7. Moretti, T.R., Koons, B.W., and Budowle, B. Enhancement of PCR amplification yield and specificity using AmpliTaq Gold™ DNA Polymerase, Biotechniques (1998) 25: 716-722.

13.8. *Forensic Science Laboratory Quality Assurance Manual* (Current Version)

13.9. *FSL Departmental Operations Manuals* (Current Versions)

13.10. *FSL Laboratory Operations Manuals* (Current Versions)

13.11. *TE Buffer*

13.12. *Organic DNA Extraction*

13.13. *Differential Organic DNA Extraction*

13.14. Quantitation by Real-Time PCR Using AB Quantifiler® Kit/7500 Instrument

13.15. Quantitiation by Real-Time PCR Using AB Quantifiler® Duo and the 7500 Instrument

13.16. Quality Control of AmpFlSTR® Identifiler® Plus PCR Amplification Kits